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ORIGINAL ARTICLE

Aminochrome Toxicity is Mediated by Inhibition of Microtubules Polymerization Through the Formation of Adducts with Tubulin

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Abstract In this study, we investigated the role of adducts formation between aminochrome and tubulin and its interference in microtubules assembly and stability in aminochrome-induced toxicity in SH-SY5Y cells. We also investigated whether changes in the microtubules structures are an early event that could affect tubulin expression. We demonstrated in vitro that aminochrome tubulin adducts inhibit tubulin polymerization and that aminochrome induces microtubules disassembly. Moreover, when the SH-SY5Y cells were incubated with aminochrome, we observed an increase in soluble tubulin, indicating depolymerization of microtubules. Aminochrome generates disruption of the microtubules network, leading to changes in the morphology of the cells inducing cell death, in a dose- and time-dependent manner. Interestingly, these changes preceded cell death and were partly inhibited by paclitaxel, a microtubule-stabilizing agent. Furthermore, we observed that aminochrome increased early tubulin expression before significant cell death occurred. Consequently, all these antecedents suggest that aminochrome toxicity is mediated by early disruption of microtubules network, where the adduct formation between aminochrome and tubulin could be responsible for the inhibition in the assembly microtubules and the loss of microtubules stability. Possibly, the early changes in tubulin expression could correspond to compensatory mechanisms against the toxic effects of aminochrome.

Introduction

Parkinson's disease (PD) is a neurodegenerative disorder involving progressive motor degeneration. A feature of this disease is the loss of dopaminergic neurons containing neuromelanin in the *substantia nigra pars compacta* (SNpc) (Lees et al. 2009). Although there are many efforts to elucidate the causes of this disease, the mechanisms responsible of dopaminergic neurons loss, are not well understood. Currently, disruption in the microtubule network is one of the mechanisms proposed to be involved in the pathogenesis of this disease (Cartelli et al. 2012).

Microtubules are major components of the cytoskeleton network participating in many cellular functions, such as cell division and intracellular trafficking, being indispensable for the survival and development of neurons (de Forges et al. 2012; Breuss and Keays 2014; Lancaster and Baum 2014). Microtubules are assembled as heterodimers of α- and β- tubulin subunits forming a highly dynamic polymer of nature. They are maintained as a dynamic equilibrium between soluble and polymerized form, where the dynamic microtubule behavior shows alternating phases of growth and shrinkage which are separated by rescue and catastrophe stages, respectively. A rescue event indicates a transition from shrinkage to growth. By contrary, a catastrophic event indicates a transition from growth to shrinkage (Gardner et al. 2013). It was reported that the

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dynamic features of microtubules would be altered by different factors. In fact, the dynamic nature of microtubules can be modified by tubulin isotypes, post-translational modifications, and also by microtubule-regulatory proteins, that ultimately would affect microtubule stability (Sirajuddin et al. 2014).

Therefore, it is known that various non-motor microtubules binding proteins regulate the stability and dynamics of microtubules (Kiris et al. 2010; Dehmelt and Halpain 2005; Sayas and Avila 2014). Moreover, the ability of different drugs to act as microtubules-disrupting agents, are currently being investigated. Thus, several microtubule-interacting drugs have been discovered or are being developed (Kaur et al. 2014). Currently, they are classified according to their action mechanism as microtubule-destabilizing or microtubule-stabilizing agents, such as vinca alkaloids and taxanes, respectively (Jordan and Kamath 2007). Likewise, some endogenous toxins have also been proposed to interfere with microtubules function, being involved in the pathogenesis of the PD (Paris et al. 2010; Huenchuguala et al. 2014).

It has been reported that over eighty-five percent of patients diagnosed with PD correspond to sporadic PD and only about fifteen percent of them could be explained by an inherited genetic component (Samii et al. 2004). The genetic forms of the disease include mutations in the *parkin*, *LRRK2*, and *α-synuclein* genes, among others. Several studies postulated that the proteins encoded by these genes, would have a crucial role in microtubules stability (Gąssowska et al. 2014; Law et al. 2014; Cappelletti et al. 2015). In addition, alterations in microtubules are observed in various PD neurotoxic models used as neurotoxin MPP (+) (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridinium ion) or rotenone. PC12 cells exposed to MPP (+) showed an axonal transport deficiency and mitochondrial damage, previous to microtubules dysfunction (Cartelli et al. 2010). Furthermore, MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced early changes in microtubules stability of dopaminergic neuron in C57Bl mice (Cartelli et al. 2013). Similarly, rotenone was also described to act by inhibiting microtubule assembly (Srivastava and Panda 2007). However, not only changes in the microtubule network were observed, but changes were also observed in the expression of proteins that form or interact with microtubules. It was reported that there is an increase in the expression of βIII-tubulin in MPTP-treated mice (Cartelli et al. 2013).

On the other hand, disturbances in axons, dendrites, and synapses are an early feature of the PD (Chu et al. 2012; Kanaan et al. 2013). Alterations in *substantia nigra* were associated with synaptic dysfunction and abnormal protein aggregation at pre-motor stages of PD, which may be triggered by disruption of the microtubules network (Ferrer et al. 2011). In the same way, epigenetic modifications in genes encoding for proteins that form microtubules or are associated with them, were also reported to be linked to PD (Masliah et al. 2013).

Nowadays, all research in the area seeks to discover the agents responsible for. It is well known that the dopaminergic neurons that degenerate in PD are those that synthesize neuromelanin (Hirsch et al. 1988; Herrero et al. 1993). This pigment accumulates through time and age (Zecca et al. 2002, 2008). Since aminochrome is the precursor of neuromelanin, this suggests that aminochrome also could be formed through time. In the present, most PD models do not explain the slow progress of this disease. Since this disease takes years to manifest the first symptoms in patients, it is proposed that the origin of the toxin could be more endogenous than exogenous. We postulated aminochrome as a possible endogenous neurotoxin responsible for neuronal degeneration in Parkinson's disease (Paris et al. 2007; Segura-Aguilar et al. 2014). Dopamine oxidizes into dopamine *o*-quinone and subsequently forms aminochrome (Segura-Aguilar and Lind 1989). An abundance of evidence shows that aminochrome could induce neurotoxicity through different mechanisms which includes formation and stabilization of α-synuclein protofibrils, dysfunction of protein degradation systems, mitochondrial dysfunction, and oxidative stress (Arriagada et al. 2004; Paris et al. 2009, 2010; Aguirre et al. 2012; Muñoz et al. 2015; Huenchuguala et al. 2014). Interestingly, all of these alterations are also involved in the mechanism of PD (Blesa et al. 2015; Gan-Or et al. 2015; Hang et al. 2015; Franco-Iborra et al. 2015; Xu et al. 2015). Also, we consider important to note that most of these mechanisms are related with microtubules networks. On the other hand, we previously have showed that aminochrome induces aggregations of α- and β-tubulin (Paris et al. 2010; Huenchuguala et al. 2014). However, we have no knowledge of the mechanism by which aminochrome affects the microtubule network and whether these effects could be an early event prior to cell death.

Therefore, the aim of this study was to determine whether aminochrome forms adduct with tubulin and if this affects the assembly microtubules and their stability, prior to significant changes in the cell death.

Experimental Procedures

Chemicals

Dopamine and tyrosinase were purchased from Sigma-Aldrich (H8502 and T3824-50KU respectively). For Viability/Cytotoxicity analysis we used calcein AM (Invitrogen, L3224) and propidium iodide (P4864, Sigma-Aldrich). Cell-

Light Tubulin-GFP (C10613) was purchased from Invitrogen. A tubulin polymerization assay kit (Cat. # BK006P), purified porcine brain tubulin (TL238), and paclitaxel (Cat. # TXD01) were purchased from Cytoskeleton. Nitro Blue Tetrazolium (NBT) (N6639) was purchased from Sigma-Aldrich. For QRT-PCR analysis we used a Brilliant III Ultra Fast SYBR Green QRT-PCR Master mix from Agilent Technologies (Cat. # 600886). We used the following antibodies obtained from Santa Cruz Biotechnology: anti-GAPDH (sc-25778), anti-rabbit IgG-AP (sc-2034), anti-mouse IgG-AP (sc-358915). We also used Anti-βIII-Tubulin from Promega (G712A). For the differentiation procedure we used retinoic acid (sc-200898) and TPA (12-O-tetradecanoylphorbol-13-acetate) (sc-202021) from Santa Cruz Biotechnology. Vinblastine was acquired from Laboratorio de Chile in the name of Lemblastine. BCIP/NBT detection kit was purchased from Invitrogen (N6547).

Cell Culture and Differentiation

The SH-SY5Y cell line was purchased from ATCC (N°ATCC. CRL-2266). The growth media used was DMEM/F12 (Dulbeccós modified medium, Hyclone sh30004.04) supplemented with 7.5 % bovine serum (Biological industries, 04-003-1B), 1,5 % fetal bovine serum (Biological industries, 04-001-1A), 1X of non-essential amino acids solution (MEM 100X, Hyclone sh30238.01), and 2X penicillin–streptomycin amphotericin B solution (Biological industries, 03-033-1B) at a concentration of 200 U/mL of penicillin G sodium salt, 0.2 mg/mL of streptomycin sulfate, and 0.5 μg/mL of amphotericin B (Cuevas et al. 2015). Cell cultures were kept in an incubator at 37 °C with an atmosphere of 5 % CO_2. When cells reached an 80 % confluence we started the differentiation of SH-SY5Y cells into dopaminergic neurons. Differentiation was carried out in two steps: in the first step, the cells were cultured for 3 days in DMEM/F12 media (supplemented with 4 % bovine serum, 1X MEM, 2X penicillin–streptomycin amphotericin B solution) containing 10 μM retinoic acid (RA). In the second stage, the cells were cultured for 3 more days in the same media but instead of retinoic acid we used 80 nM of TPA (12-O-tetradecanoylphorbol-13-acetate). After 6 days of differentiation, we carried out the treatments for 0.5, 2, 4, 9, or 16 h with 15, 30, or 50 μM aminochrome. Also, the cells were incubated with 0.05 and 0.1 μM paclitaxel in the presence or absence of 50 μM aminochrome during 16 h. Cells were treated with aminochrome in serum-free media for 1.5 h. After this time, we removed the media and replaced it for fresh growth media until incubation time was completed, except for treatment with paclitaxel which was maintained for 16 h.

Synthesis and Purification of Aminochrome

Dopamine (7.5 mmol) and 10 ng of tyrosinase were incubated in 25 mM potassium phosphate buffer pH 6 for 15–20 min at room temperature. To purify aminochrome, the incubation solution was loaded on a CM-Sephadex C50-1000 (18 × 0.7 cm) column (Sigma-Aldrich, C25120). The red–orange solution corresponding to aminochrome was collected and detected spectrophotometrically by measuring the absorbance at 480 nm. Aminochrome concentration was determined by the molar extinction coefficient of 3058 $M^{-1}cm^{-1}$ (Segura-Aguilar and Lind 1989).

Tubulin Polymerization Assay

The assay was carried out using a HTS-Tubulin Polymerization assay kit (Cytoskeleton, Cat # BK004P). Tubulin polymerization assay is based on an adaptation of the original method of Shelanski (Shelanski et al. 1973) and Lee and Timasheff (1977) which demonstrated that light is scattered by microtubules to an extent that is proportional to the concentration of microtubule polymer. The resulting polymerization curve is representative of the three phases of microtubule polymerization, namely nucleation (I), growth (II), and steady state equilibrium (III). Each assay used 3 mg/mL porcine brain tubulin (Cat # HST03) in a volume of 100μL of PEM buffer plus glycerol (80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM GTP, 10 % glycerol). Polymerizations were followed by an increase in absorbance at 340 nm over a 60 min period at 37 °C. We analyzed the polymerization of tubulin with 20 μM paclitaxel (Cytoskeleton, Cat. # TXD01), 50 μM aminochrome, and 30 μM vinblastine (Lemblastine, vinblastina 10 mg, Laboratorio de Chile).

Cell Death

For live/dead experiments, cells were cultured in 24-well dishes according to culture and differentiation protocol previously described. Cell death was determined by using calcein AM and propidium iodide, two fluorescent reagents that discriminate the population of live cells from the dead cell population. After aminochrome treatment, SH-SY5Y cells were washed with PBS 3 times for 5 min. Then, cells were incubated for 30 min in the dark with 1.5 μM of calcein AM and 1 μM of propidium iodide, both prepared in PBS. Live and dead cell counting was performed in a Leica fluorescence microscope (DM1L). We counted 100 cells per replicate.

Microtubule Extraction

After aminochrome treatment, cells were rinsed vigorously with 1 mL of PBS in order to release them. Cells were

centrifuged at 5000RPM for 10 min and the pellet was washed 2 times with PBS. Next, 25μL of PEM buffer containing 80 μM PIPES pH6.8, 1 mM EGTA, 1 mM MgCl2, 0.5 % Triton X-100, and 25 % glycerol were added to permeabilize the cells and release tubulin monomers (soluble tubulin). After 10 min of incubation, we collected the supernatant and we rinsed cells with 25μL of 2X RIPA buffer (100 mM Tris–HCl pH7.4, 300 mM NaCl, 2 % NP-40, 1 % sodium deoxycholate, 0.2 % SDS) with 1:100 protease inhibitors cocktail (Thermo Scientific, Cat. # 78430). This rinse buffer was collected with the initial extraction solution. The pellet (cell ghosts containing polymerized tubulin) was lysed with 25μL of 1X RIPA (50 mM Tris–HCl pH8, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 1 % SDS, and 1:100 protease inhibitors cocktail) with 25μL of 50 mM Tris pH8 buffer. Soluble and polymerized tubulin was stored at -80 °C for future analysis.

Quinoprotein Detection by NBT Staining

Nitro Blue Tetrazolium (NBT) staining was performed as described by Paz (Paz et al. 1991). This quinoprotein detection is based on the property that quinoproteins catalyze redox cycling in an alkaline pH in the presence of excess glycine as reductant. With NBT and oxygen present, there is concomitant reduction of the tetrazolium to formazan. Samples were first separated in denaturing conditions (SDS-PAGE) or non-denaturing conditions (NATIVE-PAGE). These were then transferred to 0.2 μm nitrocellulose membranes (Biorad, Cat. # 162-0112). Membranes were incubated in a light covered container with a 0.6 mg/mL NBT solution prepared in 2 M pH10 potassium glycinate buffer for 60 min, or until the blue-purple colored band appeared positive to quinoproteins. NBT reaction was stopped by washing the membrane with a 0.16 M sodium borate solution 3 times for 5 min each.

Western Blot

The levels of proteins expression were determined in cell lysates and in soluble and polymerized fractions obtained after microtubules extraction. For whole cell lysate, we first washed SH-SY5Y cells with PBS and then the cells were released with 1 mL of PBS by up and down motion. Cell pellet was resuspended in cold RIPA buffer (50 mM Tris–HCl pH8, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 1 % SDS, and 1:100 protease inhibitors cocktail) and incubated for 30 min in ice. Protein samples were separated by SDS-PAGE (12 % wt/vol) and in NATIVE-PAGE (8 % wt/vol) at 150 V for 1 h. The separated proteins were then transferred electrophoretically to a 0.2 μm nitrocellulose membrane at 300 mA for 60 min.

After blocking with a solution of 0.5 % skim milk in T-TBS (10 mM Tris–HCl pH7.6, 150 mM NaCl, and 0.025 % Tween 20) for 1 h, the membrane was incubated with the anti βIII-tubulin or anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibodies overnight at 4 °C in a shaker. After washing the membrane 3 times for 15 min with T-TBS, we added the corresponding alkaline phosphatase-linked secondary antibodies for 1 h at room temperature. Proteins were detected by using a BCIP/NBT detection kit (Invitrogen, N6547).

Tubulin Immunofluorescence

For tubulin labeling, we used Cell-Light BacMam 2.0 reagents that are fluorescent protein-signal peptide fusions. This cellular labeling utilizes BacMam technology, which uses a baculovirus as a vehicle to deliver and express genes in mammalian cells. For this experiment, cells were cultured in 25-mm coverslips, and then 20μL of Cell-Light Tubulin-GFP diluted in 1 mL of growth media (DMEM F12 supplemented with 7.5 % bovine serum, 1,5 % fetal bovine serum, 1X of MEM 100X, and 2X penicillin–streptomycin amphotericin B solution) was added directly to cells, for 24 h at 37 °C and 5 % CO_2 atmosphere. First we analyzed the transduction efficiency and then we selected cells for capturing the images in an inverted fluorescence microscope (Carl Zeiss, Götintingen. Germany; model Axio Observer.Z1) for a period of 2 h. For morphology analysis, we counted approximately 100 cells by replicate.

Quantitative Real-Time PCR

The total RNA from SH-SY5Y cells was extracted with TRIZOL reagent (Invitrogen, 15596-026) according to the manufactureŕs protocol and was quantified using Nanodrop 3300 (Thermo). The cDNA was synthesized using oligo-dT (IDT) and epicenter RT reagents according to the manufactureŕs instructions. Comparative quantitation real-time PCR for the *TUBB3* gene (Fw: 5′-CGAAGCCAG CAGTGTCTAAA-3′; Rv:5′-GCTGCAATAAGACAGA GACAGG-3′) was performed in triplicate using Brilliant III Ultra Fast SYBR Green QRT-PCR Master mix in a Stratagene Mx3000p Detection system. The mRNA levels were normalized through the housekeeping gene *GAPDH* (Fw: 5′-GCCAAAAGGGTCATCATCTC-3′; Rv: 5′-TGT GGTCATGAGTCCTTCCA-3′). The following experimental run protocol was used: 25 °C for 1 s, 50 °C for 10 min, 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and a last segment that includes 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s. Mx3300P Software was used to analyze real-time data.

Statistical Analysis

All data were expressed as the mean ± SEM values. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test.

Results

### Adducts Formation Between Aminochrome and Tubulin in Vitro

In order to determine whether aminochrome forms adducts with tubulin, we performed NBT staining and posterior tubulin immunoblot (IMM). In these experiments we quantified the NBT/IMM ratio and we observed that aminochrome formed adducts with tubulin in a concentration-dependent manner (Fig. 1a, b). A significant increase in the formation of adducts was observed in presence of 15, 30, and 50 μM of aminochrome compared with control without aminochrome (0.54 ± 0.04, $P < 0.001$; 0.71 ± 0.08, $P < 0.001$; 0.76 ± 0.01, $P < 0.001$; respectively). However, we did not observe significant differences when the samples were incubated at different times (Fig. 1c, d).

Adducts Formation Between Aminochrome and Microtubules in Vitro

With the purpose of determining whether aminochrome could form adducts not only with tubulin, but also with preformed microtubules, we performed NBT staining for microtubules. The preformed microtubules were incubated in the presence of 15, 30, and 50 μM of aminochrome during 0 and 60 min, generating several bands with molecular weight over 70 kDa which seems to be oligomers corresponding to dimeric, trimeric, tetrameric, and oligomeric tubulin structures (Bands A–G, Fig. 2a). A significant increase in the formation of aminochrome adducts with microtubules was observed in the presence of 15, 30, and 50 μM of aminochrome (13 ± 0.8 %, $P < 0.001$; 16 ± 0.5 %, $P < 0.001$; 20 ± 0.4 %, $P < 0.001$; respectively) compared with the control incubated with paclitaxel (3 ± 1.5 %). These results revealed that aminochrome formed adducts with microtubules in a concentration-dependent manner (Fig. 2b).

The formation of adducts was only observed when the microtubules were treated with aminochrome alone, but not in the presence of vinblastine or paclitaxel, indicating that the NBT reaction is specific for quinones. Vinblastine and paclitaxel were used as polymerization controls, since vinblastine and paclitaxel act as microtubules-destabilizing and stabilizing agents, respectively.

Fig. 1 Aminochrome adducts formation with tubulin in a concentration-dependent manner in vitro. Tubulin (T) was incubated with 15, 30, and 50 μM aminochrome (AM) for 60 min (**a**) and 50 μM aminochrome (AM) for 0, 15, 30, and 60 min (**c**) at 37 °C. **a** NBT staining and immunoblot (IMM) at different concentrations of AM. **b** NBT/IMM ratio graph for pixels in **a**. **c** NBT staining and immunoblot (IMM) at different incubation times with AM. **d** NBT/IMM ratio graph for pixels in **c**. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (*$P < 0.05$)
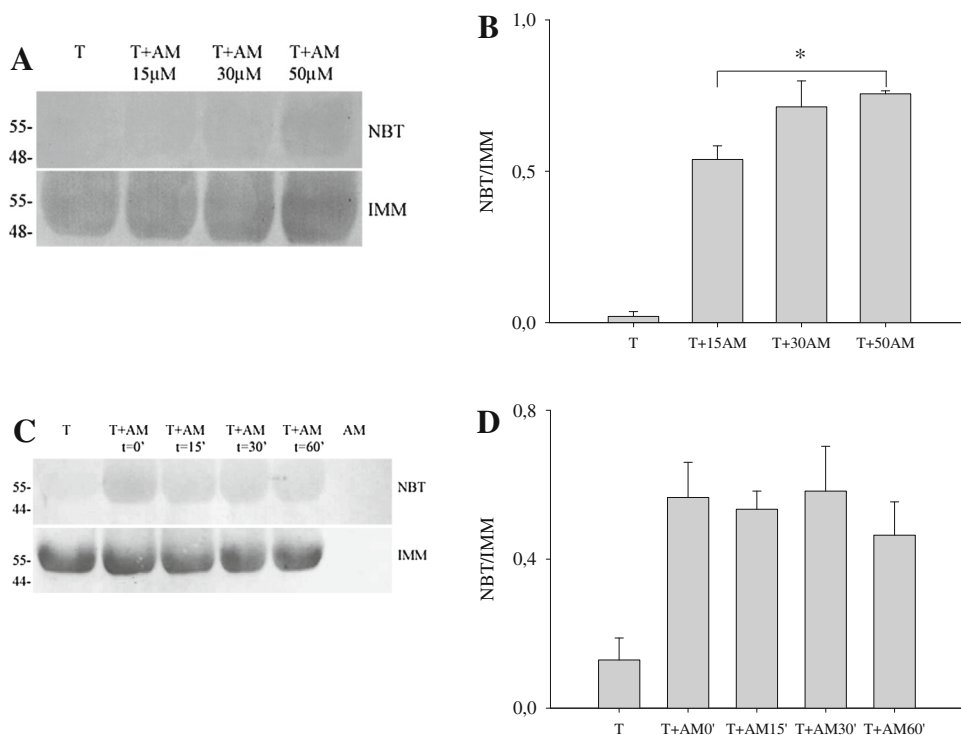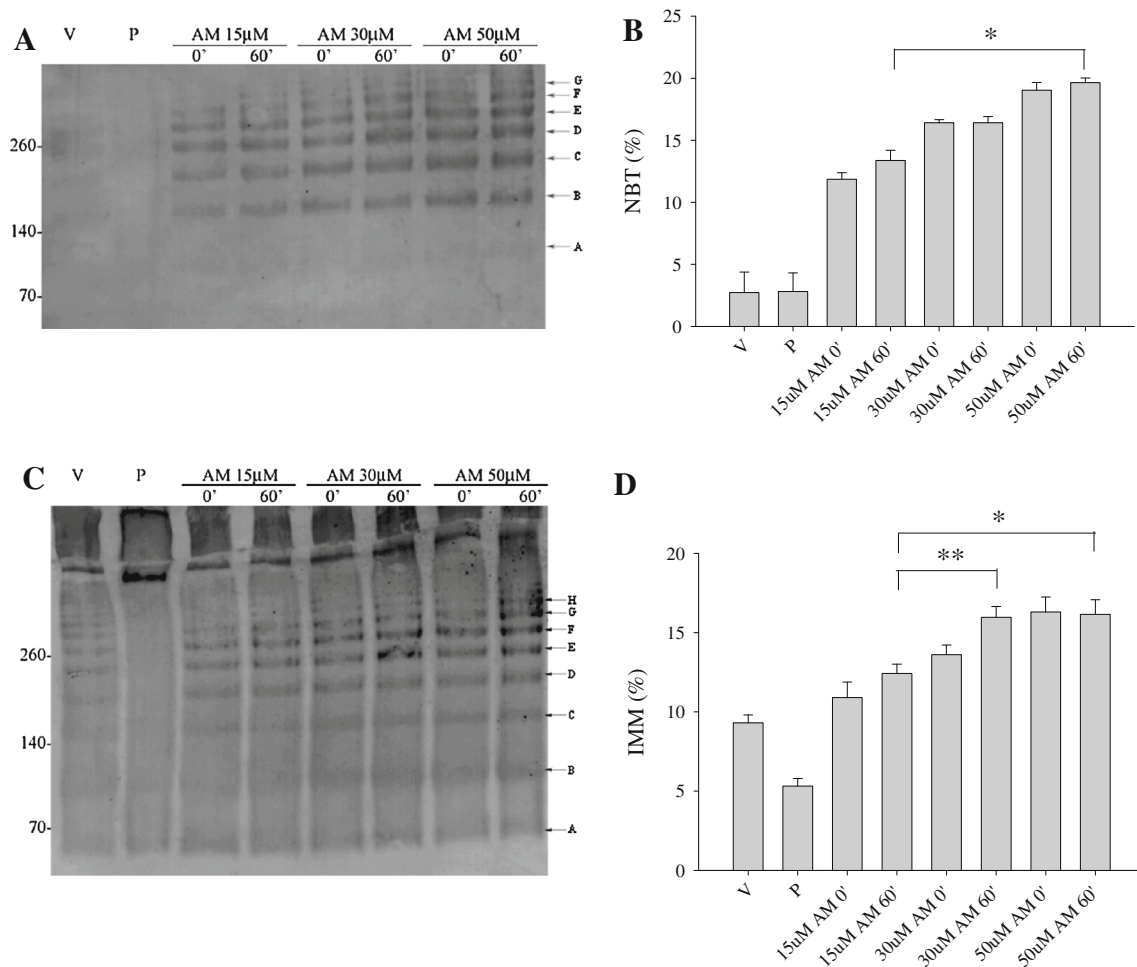
Fig. 2 Aminochrome adducts formation with microtubules in a concentration-dependent manner in vitro and microtubules depolymerization in the presence of aminochrome. Preformed microtubules were treated with 15, 30, and 50 μM aminochrome (AM), 180 μM vinblastine (V), and 200 μM paclitaxel (P) for 0 and 60 min at 37 °C. Samples were then separated in non-denaturing conditions (NATIVE 8 % wt/vol). **a** NBT staining. **b** Graph for pixels analysis of NBT bands (NBT %) in **a**. **c** Immunoblot for tubulin (IMM). **d** Graph for pixels analysis of IMM bands (IMM %) in **c**. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (*$P < 0.05$, **$P < 0.01$)

Microtubules Depolymerization in the Presence of Aminochrome in Vitro

We performed tubulin immunoblot in the same membrane used for NBT reaction to demonstrate that aminochrome induces microtubules disassembly. The presence of immunoblot bands (Bands A to H) that could correspond to sizes of tubulin mono- and polymeric structures (55, 110, 165, 220, 275, 275, and >275 KDa), clearly suggest that aminochrome induces microtubules depolymerization as observed with the microtubules-depolymerizing agent vinblastine (Fig. 2c). We also observed that aminochrome increases the percentage of pixels corresponding to tubulin immunoblot bands in a concentration-dependent manner (Fig. 2d). In contrast, we showed that paclitaxel effectively stabilizes microtubules since mainly a single larger band

was observed. A significant 2.0-fold ($P < 0.001$), 2.6-fold ($P < 0.001$), and 3.1-fold ($P < 0.001$) increase in the percentage of pixels corresponding to immunoblot bands were observed in presence of 15, 30, and 50 μM of aminochrome compared with the control paclitaxel, respectively (Fig. 2d).

Microtubules Depolymerization in SH-SY5Y Cells in the Presence of Aminochrome

In order to evaluate whether the microtubules depolymerization induced by aminochrome was also present in SH-SY5Y cells, we incubated cells in the presence and absence of 50 μM of aminochrome. Later, we obtained polymerized and soluble protein fractions for both conditions and performed tubulin immunoblot in denaturing conditions.

We observed that aminochrome increased soluble tubulin (Fig. 3a). A significant 5.5-fold ($P < 0.01$) increase in the ratio of soluble and polymerized tubulin was observed in the presence of aminochrome (Fig. 3b). Similarly, we performed tubulin immunoblot in non-denaturing conditions and we observed that aminochrome increased tubulin in the soluble protein fraction (Fig. 3c). A significant 4.2-fold ($P < 0.05$) increase in the ratio of soluble and polymerized tubulin was observed in the presence of aminochrome (Fig. 3d).

Inhibition of Tubulin Polymerization in Vitro by Aminochrome

We found previously that aminochrome forms adducts with tubulin and microtubules, destabilizing the latter and promoting their depolymerization, both in vitro as in SH-SY5Y cells. Therefore, we investigated whether aminochrome not only induces microtubules depolymerization but also affects directly the microtubules assembly. Consequently, we performed a tubulin polymerization assay following the reaction at 340 nm through time. We can distinguish in Fig. 4a three phases of polymerization: I (nucleation), II (growth), III (steady state). We observed standard polymerization reactions alone and in the presence of 20 μM paclitaxel or 30 μM vinblastine. Also, aminochrome significantly 2.4-fold decreased the polymerization rate value ($P < 0.001$) of the growth phase and generated a 28 % reduction ($P < 0.01$) in final polymer mass. On the other hand, paclitaxel eliminated the nucleation phase and enhanced 1.8-fold the polymerization rate. Conversely, vinblastine decreased by 2.4-fold ($P < 0.001$) the polymerization rate and generated a 63 % reduction ($P < 0.001$) in final polymer mass (Fig. 4b).

Aminochrome Effect on Morphology and Cell Death in SH-SY5Y Cells

A linear significant increase in cell death was observed when SH-SY5Y cells were incubated in the presence of 15, 30, and 50 μM aminochrome during 2, 4, 9, and 16 h ($P < 0.05$, $P < 0.01$, $P < 0.001$) (Fig. 5a). This effect was concentration- and time-dependent. As stated previously, aminochrome destabilizes microtubules by promoting their depolymerization. We investigated whether cell death induced by aminochrome could be inhibited by paclitaxel, a microtubule-stabilizing agent. A significant decrease in
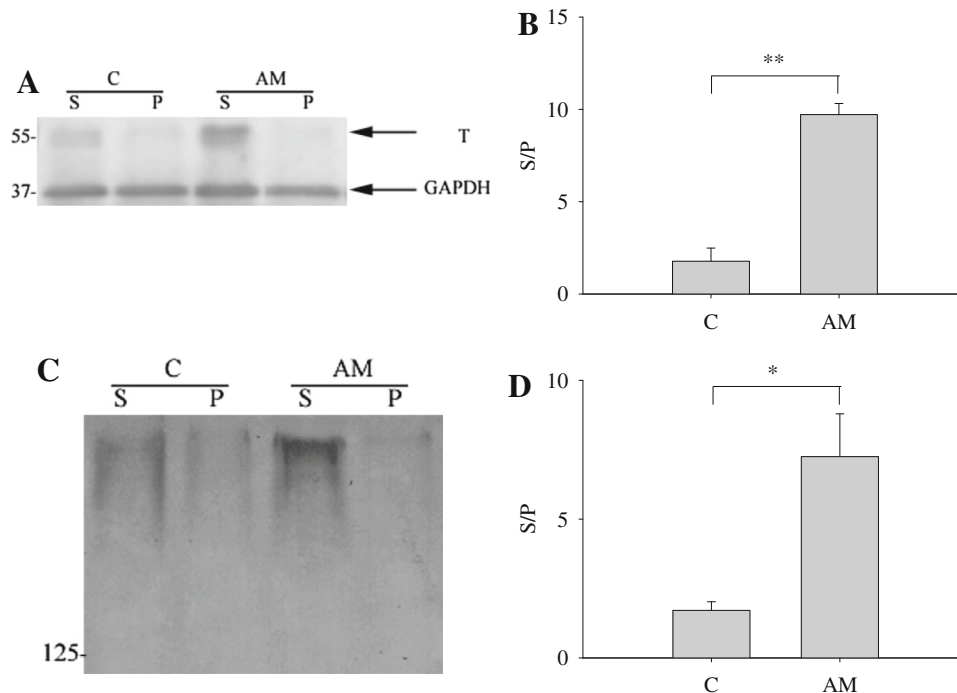

Fig. 3 Microtubules depolymerization in SH-SY5Y cells in the presence of aminochrome. The SH-SY5Y cells were incubated with 50 μM aminochrome (AM) for 16 h at 37 °C and 5 % CO_2. As control, the cells were incubated alone in culture medium (C). We performed immunoblot for tubulin in both, separated the soluble (S), and polymerized (P) tubulin fractions as was described in methodology. As a loading control we used GAPDH. **a** Immunoblot for tubulin in denaturing conditions. **b** Graph for pixels analysis of ratio of soluble and polymerized tubulin in **a**. **c** Immunoblot for tubulin in non-denaturing conditions. **d** Graph for pixels analysis of ratio of soluble and polymerized tubulin in **c**. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (*$P < 0.05$, **$P < 0.01$)
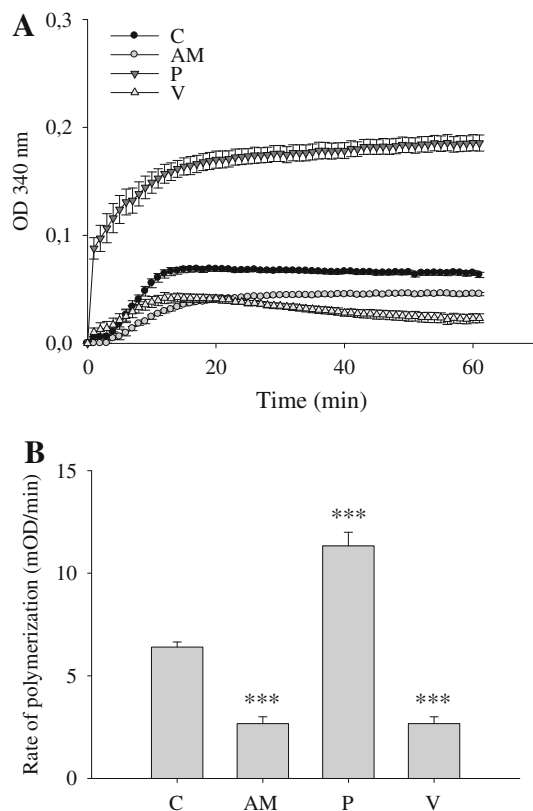
Fig. 4 Inhibition of tubulin polymerization in vitro by aminochrome. **a** A tubulin polymerization assay was carried out with 3 mg/mL of pure tubulin. We measured the effect of 50 μM aminochrome (AM), 20 μM paclitaxel (P), and 30 μM vinblastine (V) on tubulin polymerization during 60 min. We recorded the absorbance at 340 nm every 1 min at a constant temperature of 37 °C. **b** Rate of polymerization (mOD/min) was calculated as the slope of the phase II of each curve. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (***$P < 0.001$)

cell death was observed when the cells were incubated with 50 μM aminochrome in the presence of 0.05 μM paclitaxel during 16 h (19.9 %; $P < 0.01$), compared with the cells treated with aminochrome alone (28 %; $P < 0.001$) (Fig. 5b). Also, 0.1 μM paclitaxel had no effect on aminochrome-induced cell death. On the contrary, paclitaxel increases significantly cell death by itself compared with control (23.4 %; $P < 0.001$).

On the other hand, we examined the status of the microtubules network using fluorescence microscopy. When the cells were treated with 100 μM aminochrome for 2 h, we observed cellular morphology changes (Fig. 5c). Interestingly, we observed that the morphology changes precede cell death, since a significant increase in the percentage of cells with altered microtubules network (76 ± 3 %; $P < 0.001$; Fig. 5d) was observed when cell death number did not exceed 15 ± 1 % to 2 h ($P < 0.001$) (Fig. 5a).

Increased Tubulin Expression in SH-SY5Y Cells by Aminochrome

In this study, we observed that the alterations in the microtubule network are early events preceding cell death. In this sense, we determined to study if there are also early changes in the expression of *TUBB3*. These changes in protein expression may relate to compensatory mechanisms against cell death. Thus, we observed that when cells were incubated in the presence of 50 μM aminochrome, a significant 7-fold ($P < 0.05$; Fig. 6a) increase in the gene expression *TUBB3* was observed at 30 min. Similarly, a significant 4.7-fold ($P < 0.05$; Fig. 6b) and 4.8-fold ($P < 0.05$; Fig. 6b) increase was observed in the expression of tubulin to 4 h with respect to incubation to treatments of 30 min and 9 h, respectively.

Discussion

Microtubules are the most abundant constituents of the cytoskeleton network. They serve many functions in the cell, for example, allowing movement and distribution of organelles, cell division, and polarity cell (Alberts et al. 2014). Microtubules are dynamic structures fluctuating constantly between a polymerized form and a soluble form. Either alteration of this equilibrium could lead to significant damage in the cells. Early changes in cytoskeletal network can affect various cellular processes, and appear to be one of the initial events in the development of some neurodegenerative diseases, such as PD (Cartelli et al. 2010, 2013). In previous work, we demonstrated that aminochrome induced aggregation of tubulin (Paris et al. 2010), but we did not have clear evidence as to whether these aggregates are effective adducts between aminochrome and tubulin/microtubules. Proteomic studies performed with isolated rat brain mitochondria and dopamine *o*-quinone, the precursor of aminochrome, revealed the formation of adducts with tubulin, but these adducts were not found when SH-SY5Y cells were incubated with dopamine *o*-quinone (Van Laar et al. 2009).

We do not know those chemical groups that could be involved in the formation of adduct with aminochrome. However, various researches support the idea that thiols may play a role important in the assembly of microtubules, and thus could be an important target for covalent modifications (Törnqvist et al. 2002; LaVoie et al. 2005; Neely et al. 2005). Tubulin protein is one of the major proteins in brain, representing 3–4 % of the total protein content in the cells—attaining up to 20 % of all brain protein (Dráber and Dráberová 2012). Therefore, this protein becomes an important target for the action of many toxins. In this work, we observed that aminochrome formed adducts with
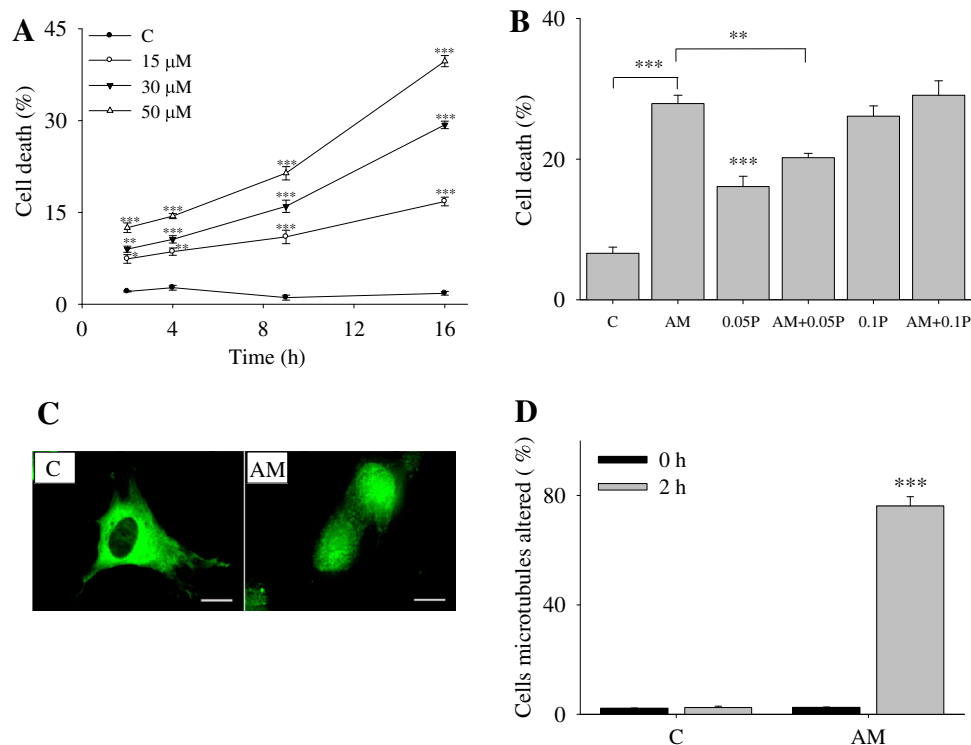
Fig. 5 Aminochrome effect on morphology and cell death in SH-SY5Y cells. **a** Quantification of the cell death. SH-SY5Y cells were treated with 15, 30, and 50 µM aminochrome (AM) for 2, 4, 9, and 16 h at 37 °C. Posterior, the cells were incubated with 1.5 µM calcein AM and 1 µM propidium iodide for 30 min in the dark and the dead and viable cells were counted. **b** Quantification of the cell death in the presence of paclitaxel. SH-SY5Y cells were treated with 50 µM aminochrome, 0.05 µM paclitaxel (P), 0.1 µM paclitaxel (P), 50 µM aminochrome plus 0.05 µM paclitaxel (P), 50 µM aminochrome plus 0.1 µM paclitaxel (P) for 16 h at 37 °C. As control, the cells were incubated alone in culture medium (C). Posterior, the cells were incubated with 1.5 µM calcein AM and 1 µM propidium iodide for 30 min in the dark and the dead and viable cells were counted. **c** Tubulin immunofluorescence images. Cells infected previously with Cell-Light Tubulin-GFP, were treated with 100 µM aminochrome for 2 h at 37 °C. **d** Quantification of cells with altered microtubule network. We measured the number of cells with altered microtubule network before and after incubation with aminochrome. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (**$P < 0.01$, ***$P < 0.001$)

tubulin and microtubules in vitro in a concentration-dependent manner. The fact that there are no differences observed in the incubation time could suggest us that the formation of adducts with microtubules or tubulin may be a very rapid reaction, although for evaluation of this reaction rate, further study would be necessary.

Possibly, adduct formation between aminochrome and tubulin could affect directly the interactions between tubulins that form the dimer required for microtubules formation. Similarly, adduct formation between aminochrome and microtubules could also affect interactions between tubulin along the microtubule protofilament that maintains their stability. Moreover, many proteins interact with the microtubule and some of them could be responsible for both, stability and dynamics of this polymer (Dráber and Dráberová 2012; Sayas and Avila 2014; Kamath et al. 2010). Since aminochrome is capable of forming adducts with both tubulin and microtubule, this could interfere with binding of microtubule-stabilizing proteins, as Tau and MAP, and

destabilize this polymer, favoring its depolymerization. The MAPs family, including MAP2 and Tau, are proteins involved in regulating microtubule stability (Kiris et al. 2010; Feinstein and Wilson 2005). These proteins were found mainly in neurons (Dehmelt and Halpain 2005). Likewise, aminochrome could interfere with exchange between GDP and GTP on β-subunits, affecting the assembly of microtubules. Moreover, the formation of adducts between aminochrome and microtubules could destabilize GTP bound to β-tubulins, favoring GTP hydrolysis or destabilizing plus end microtubules or microtubule protofilaments. Microtubules that have GDP-tubulin at their ends are susceptible to be disassembled (Dráber and Dráberová 2012). Therefore, these effects can lead to the loss of microtubule stability and consequently to their depolymerization. In this work, the fact that the bands observed in the adduct formation correspond to tubulin equivalent to dimeric, trimeric, tetrameric, or multimeric structures, indicates that aminochrome not only forms
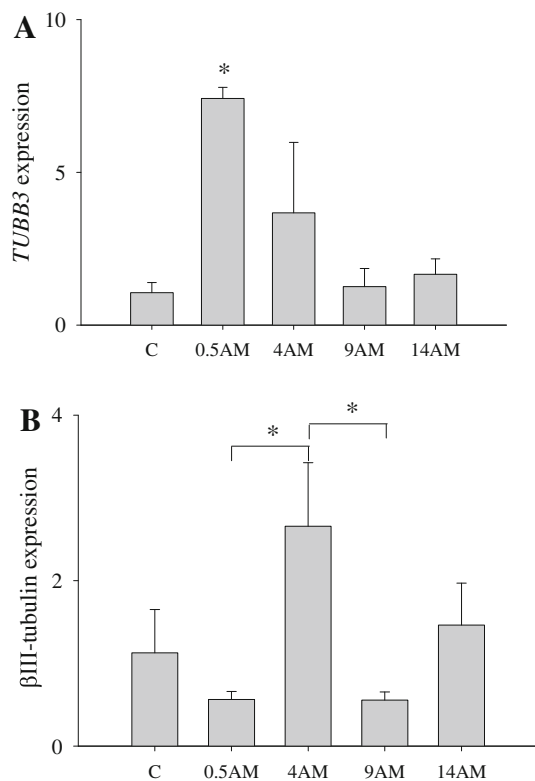
Fig. 6 Tubulin expression in SH-SY5Y cells by aminochrome. **a** Quantitative real-time PCR. Total RNA from SH-SY5Y cells treated with 50 μM aminochrome for 0.5, 4, 9, and 14 h for 37 °C and 5 % CO_2, was extracted as described under experimental procedures. A comparative QRT-PCR performed for *TUBB3* gene revealed that aminochrome increases the expression of *TUBB3*. mRNA levels were normalized through the housekeeping gene *GAPDH*. **b** The graphic shows the relative expression of tubulin. The statistical significance was assessed using analysis of variance (ANOVA) for multiple comparisons and Newman–Keuls test (*$P < 0.05$)

microtubules mass in vitro. This suggests that microtubule binding sites, among them the vinca domain and colchicine binding site, could be involved.

The vinca site is situated near the exchangeable GTP binding site on β-tubulin of the αβ-heterodimer. Microtubule-interacting drugs in this site, like vinblastine, can bind at tubulin dimers, at the plus end of the microtubule or along the microtubule protofilaments (Gigant et al. 2009; Risinger and Mooberry 2012; Kaur et al. 2014). This can decrease the rate of microtubule growth and destabilize microtubules, which could inducethe disassembly of microtubules and diminution in the microtubules mass. Given that aminochrome has an effect similar to that of vinblastine, this suggests the possibility that the aminochrome effect involves binding at the vinblastine binding site on tubulin or others sites. This could finally interfere with the ability of tubulin dimer to polymerize. The colchicine binding site, present in the interface between α-tubulin and β-tubulin, is also related with microtubules depolymerization (Kaur et al. 2014). That site could also be a target of aminochrome action. All these results suggest to us that aminochrome through adduct formation with tubulin not only induces disassembly of microtubules, but also inhibits their polymerization.

Many drugs may destabilize the microtubules network and induce cell death (Choi et al. 2014; Kamal et al. 2014). Thus, vinblastine-induced microtubules destabilization enhances aminochrome-induced cell death (Paris et al. 2011). Microtubules destabilization was observed in genetic and idiopathic Parkinson's disease patient fibroblasts. The parkinsonians fibroblasts present a higher fraction of unpolymerized tubulin in respect to control cells (Cartelli et al. 2012). Microtubules destabilization was also observed in a PD model (Ren et al. 2005; Choi et al. 2011).

Aminochrome was toxic in SH-SY5Y cells. Interestingly, before observing significant changes in cell death, we observed that most cells exhibited major modifications in cell morphology indicating disruption in the microtubules network. Since the changes in microtubule stability could be responsible for cell death, we evaluated aminochrome toxicity in the presence of the microtubules-stabilizing agent, like paclitaxel. Paclitaxel acts by inhibiting the toxic effects of microtubules-destabilizing agents (Zajkowski et al. 2015; Hongo et al. 2012). But, a high degree of stabilization of microtubules could also be harmful for cell (Baas and Ahmad 2013; Gornstein and Schwarz 2014). Interestingly we observed that cell death can be reversed in part by paclitaxel, suggesting that stability in the microtubules network could be an important event in PD pathogenesis or in other neurologic and neurodegenerative disorders (Li et al. 2003; Lou et al. 2014).

Therefore, aminochrome toxicity seems to be mediated by early changes in microtubules stability. Our results

adducts with tubulin, but aminochrome could also affect microtubule stability inducing their depolymerization.

Similarly, we observed in the immunoblot for tubulin the presence of various bands of a size proportionate to tubulin, suggesting that aminochrome effectively induced depolymerization of microtubules in vitro. Likewise, the depolymerization of microtubules or the inhibition of their polymerization was observed when the SH-SY5Y cells were incubated in the presence of aminochrome, indicating an increment of tubulin in the soluble fraction and diminution in the polymerized fraction. This suggests microtubules depolymerization and/or inhibition of the polymerization in cell culture. It is possible that both processes may be present, contributing to a decrease in mass of microtubules. While we do not know the exact mechanism leading to these effects, possibly the formation of adducts between aminochrome with tubulin/microtubules could be a key factor.

Moreover, we showed that aminochrome diminished the rate of polymerization of microtubules and decreased

showed that the changes in cell morphology with altered microtubules network are accompanied by increased expression of tubulin before aminochrome-induced cell death is observed. These results lead us to postulate that changes in the expression of tubulin are intended to reverse the toxic effect induced by aminochrome on microtubules network. The polymerization of microtubules takes place above a critical concentration of tubulin dimers. Since aminochrome was able to form adducts with tubulin, this effect would apparently decrease tubulin dimers concentration required to polymerize. Therefore, increased expression of tubulin could be a compensatory mechanism against aminochrome toxicity. Augmented expression of tubulin has been observed in models of PD (Cartelli et al. 2013).

PD is a multifactorial disease that has been related not only with alterations in cytoskeleton network, but also with the formation and stabilization of α-synuclein neurotoxic oligomers, dysfunction of protein degradation systems, mitochondrial dysfunction, axonal dysfunction, reticulum stress, and oxidative stress (Blesa et al. 2015; Gan-Or et al. 2015; Hang et al. 2015; Franco-Iborra et al. 2015; Xu et al. 2015; Hongo et al. 2012; Esteves et al. 2014; Lu et al. 2014; Segura-Aguilar et al. 2014; Segura-Aguilar and Kostrzewa 2015). Alterations in cytoskeleton networks appear to be involved in most of these mechanisms where microtubules play a role. In fact, microtubules-stabilizing agents were proposed as therapeutics alternative against neurodegenerative disease (Brunden et al. 2014; Lou et al. 2014). On the other hand, some authors indicate that the axonal transport alteration precedes the formation of aggregates (Janezic et al. 2013). Since axonal transport is dependent on microtubules, this suggests that the alterations in microtubules network could be an event early in the progression of this disease. Possibly, the initial changes in the microtubules network could represent the critical event that eventually triggers a series of consecutive events leading to cell death of dopaminergic neurons. Microtubule dysfunction was observed in fibroblasts of PD patients who showed a reduced microtubule mass (Cartelli et al. 2012).

Since aminochrome could form adducts with several proteins, the fact that tubulin is found in high concentrations in the cell, might suggest that the reaction of aminochrome is favored toward the formation of adducts with tubulin, possibly more than the formation of neuromelanin or the reaction with other proteins. It is difficult to discern which of these events prevails, since the reaction may depend in part on protein concentration, aminochrome concentration, stability of adduct, turnover rates of the protein, and the constant associated with the formation of adducts with different proteins (Törnqvist et al. 2002). If the reaction of aminochrome with tubulin is faster than the neuromelanin polymerization or others reactions with

proteins, it is unknown. One feature of PD is its slow and gradual deterioration. Neurons that are lost in this disease synthesize neuromelanin over time; and aminochrome formation is a requisite (Lees et al. 2009). The alterations in the microtubule network could be an early and cumulative event that in part is responsible for cell death induced by aminochrome. Moreover, this could mark the beginning of a series of events leading irreversibly to cell death. Although microtubule stabilization decreased aminochrome-induced cell death, inhibition was incomplete. This suggests that simultaneously different mechanisms may be operating. But which of these mechanisms predominates would be determined by the ability to interact aminochrome with different proteins and the abundance of these proteins in the cell. Since tubulin is a protein very abundant in the cell, this might suggest that plays a predominant role in the development of this disease.

In conclusion, our results suggest that aminochrome toxicity in SH-SY5Y cells could be mediated in part by early disruption of microtubules networks, where adduct formation between aminochrome and tubulin could be responsible for the inhibition of assembly microtubules and the loss of microtubules stability. It is particularly notable that aminochrome inhibits assembly and/or increases disassembly of microtubules, since this would affect early diverse cellular processes, among them, organelles distribution in the cell, transport and formation of vesicles in secretory pathway, and fusing aggresomes/autophagosome with lysosomes. Given that changes in microtubules network preceded cell death and may be partly inhibited by microtubule-stabilizing agents, this supports the concept that microtubules play a key role in cell survival. More so, early changes in tubulin expression could correspond to compensatory mechanisms against the toxic effects of aminochrome.

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